Article No. jmbi.1999.2982 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (1999) 291, 693–701

JMB

The Volume and Compressibility Changes of Lysozyme Associated with Guanidinium Chloride and Pressure-assisted Unfolding

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The apparent specific volumes and isentropic compressibilities of hen egg white lysozyme were measured in aqueous guanidinium chloride solutions at 25 °C by means of a vibrational densimeter and a sing-around ultrasonic velocimeter. Little transition attributable to a protein unfolding was detected in the partial specific volume, while the partial specific isentropic compressibility decreased slightly around the transition region. The pressure-assisted unfolding was also investigated in aqueous guanidinium chloride solutions by means of ultraviolet spectroscopy. Assuming a two-state transition model, it was found that the free energy change of unfolding depends almost linearly on pressure and the unfolding reaction is accompanied by a small decrease in volume. The compressibility behavior is in conflict with the notion that a protein structure is almost completely unfolded by guanidinium chloride and most of the amino acid residues in the protein interior are exposed to solvent. These results support the current view that globular proteins have some residual structures even in the unfolded state induced by a strong denaturant.

Keywords: hen egg white lysozyme; guanidinium chloride; partial specific volume; partial specific isentropic compressibility; pressure-assisted unfolding

Introduction

A detailed understanding of the structural and thermodynamic properties of the unfolded states of proteins has been a major topic of research in the field of protein chemistry (Dill & Shortle, 1991; Shortle, 1996). It has been demonstrated that proteins are extensively unfolded in the presence of a strong denaturant such as guanidinium chloride (GdnCl) and the unfolded proteins have hydrodynamic properties expected for a random-coil polypeptide chain (Tanford, 1968).

The partial specific isentropic compressibility is an important physical quantity directly related to the conformation state of protein molecule, because it involves the contributions of the more compressible atomic contacts due to imperfect packing of the polypeptide chain in the protein interior and the less compressible surface hydration (Chalikian & Breslauer, 1996a). Therefore, study on the compressibility behavior is of interest in order to elucidate the surface hydration behavior in connection with the protein unfolding process. However, only limited data have been reported on the compressibility change associated with protein unfolding. Chalikian *et al*. (1995, 1996a) reported that the acid and base-induced transitions of cytochrome *c* are accompanied by small decreases of −0.03 to −0.07 $cm^3g^{-1}GPa^{-1}$ in compressibility. They have interpreted these small decreases in compressibility as being a result of the transition from native to partially unfolded states (about 70% of solvent-accessible surface area expected for a fully extended chain) of globular proteins.

On the other hand, Gekko and his co-workers (Tamura & Gekko, 1995; Kamiyama & Gekko, 1997) reported that the GdnCl-induced unfolding of ribonuclease A and hen egg white lysozyme brings about large decreases of −0.15 to −0.16 $cm^3g^{-1}GPa^{-1}$ in partial specific isentropic compressibility and decreases of −800 to −1100 $cm^3\ mol^{-1}$ in partial molar volume. This

Abbreviations used: GdnCl, guanidinium chloride; CD, circular dichroism.

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0022-2836/99/330693–9 $30.00/0

compressibility reduction is in agreement with the theoretical value for the completely unfolded state calculated by assuming the empirical additivity scheme of isentropic compressibilities of extended oligopeptides and polypeptides (Kharakoz, 1997). Gekko *et al*. have interpreted these large decreases in compressibility as being a result of the transition of globular proteins from native to almost completely unfolded states, resulting in the exposure of a large number of amino acid residues to solvent. Thus, it seems that the GdnCl-induced unfolded state is in contrast to the partially unfolded state by acid or base-induced transitions (Chalikian & Breslauer, 1996a, 1998).

It should be pointed out, however, that the decreases in volume for GdnCl-induced unfolding reported by Kamiyama & Gekko (1997) are surprisingly greater than the earlier literature values (Lee & Timascheff, 1974; Skerjanc & Lapanje, 1972; Li *et al*., 1976; Samarasinghe *et al*., 1992; Hayashi *et al*., 1996). Furthermore, recent studies have elucidated that the unfolded state, even in 6 M GdnCl, is not necessarily modeled by a random-coil polypeptide chain (Dill & Shortle, 1991; Shortle, 1996). Therefore, it is necessary to re-examine more precisely the volume and compressibility behavior of globular proteins in denaturant solutions. Here, we examine the volume and compressibility changes of lysozyme associated with GdnCl-induced unfolding in order to characterize the chemically unfolded states.

Results

Figure 1 shows the plots of the native fraction of lysozyme estimated from ellipticities at 289 nm and at 222 nm by circular dichroism (CD) measurement and from $d\varepsilon/d\lambda$ values at 290 nm by UV measurement as a function of GdnCl concentration at 25 °C. The $d\varepsilon/d\lambda$ values were calculated from intensity, $dA/d\lambda$, at 290 nm of first-order differential absorption spectra of lysozyme where A is absorbance, ε ($mol^{-1}cm^{-1}$) is molar extinction coefficient of the protein and λ (nm) is wavelength. As revealed by the native fraction, the tertiary and secondary structures of lysozyme cooperatively unfold around the transition region.

Apparent specific volume, ϕ_v, and apparent specific isentropic compressibility, ϕ_k, of lysozyme in solution were calculated from the following equations (Iqbal & Verrall, 1987):

$$\phi_v = \frac{1}{w}\left(\frac{1}{d} - \frac{1}{d_0}\right) + \frac{1}{d_0} \tag{1}$$

$$\phi_k = \frac{1}{w}\left(\frac{\beta}{d} - \frac{\beta_0}{d_0}\right) + \frac{\beta_0}{d_0} \tag{2}$$

where d and β are the density and the coefficient of isentropic compressibility of the solution, respectively, w is the mass fraction of the protein, and the subscript 0 refers to the solvent. The coefficient β is estimated from the sound velocity, u, and the density, d, measurements by using:

$$\beta = 1/(u^2 d) \tag{3}$$

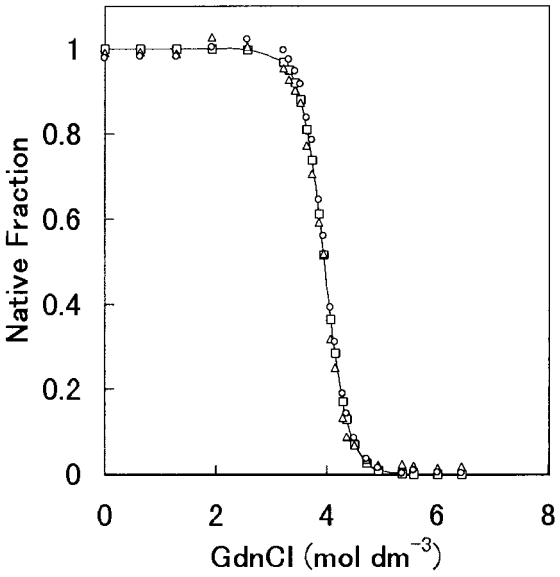

Figure 1. Native fraction of lysozyme estimated from the ellipticities at 289 nm and at 222 nm by CD measurement and from the $d\varepsilon/d\lambda$ at 290 nm by UV measurement as a function of GdnCl concentration at 25 °C. Circle, CD 289 nm; triangle, CD 222 nm; square, UV.

The dependence of ϕ_v or ϕ_k values on lysozyme concentration was evaluated in various GdnCl solutions at 25 °C. For dilute protein solutions less than 0.3 wt%, the variation in ϕ_v and ϕ_k values on concentration can be adequately represented by the following linear relations (4) and (5), respectively:

$$\phi_v = \phi_{v^0} + S_v w \tag{4}$$

$$\phi_k = \phi_{k^0} + S_k w \tag{5}$$

where ϕ_{v^0} and ϕ_{k^0} are the infinite dilution values, which are equal to the limiting partial specific volume, v^0, and isentropic compressibility, k^0, respectively, and S_v and S_k are the experimental slopes.

The ϕ_v and ϕ_k values were fitted to equations (4) and (5) by the least-squares method outlined earlier (Sakurai *et al*., 1994). The limiting v^0 and k^0 values in aqueous solution without denaturant are listed in Table 1 and compared with the literature values. The v^0, k^0, S_v, and S_k values in aqueous GdnCl solutions are summarized in Table 2 along with their standard deviations. Figure 2 provides the plots of the v^0 and k^0 values of lysozyme as a function of the GdnCl concentration. While the

Table 1. Partial specific volume and isentropic compressibility of lysozyme at 25 °C

Solvent	pH	v^0 (cm^3 g^{-1})	k^0 (cm^3 g^{-1} GPa^{-1})
Water[a]		0.734(0.001)	0.071(0.009)
Water-phosphate[b]	7	0.712	0.033
Water[c]	6.65	0.742	0.091
Water-HCl[d]	4	0.709(0.001)	0.033
Water-KCl-HCl[e]	3	0.738	0.068
Water[f]		0.699	0.013
Water-KCl-HCl[g]	3	0.702(0.001)	
Water-KP$_i$[h]	2.5-3.5	0.725	

Numbers in parentheses denote standard deviations.
[a] This study.
[b] Gekko & Noguchi (1979).
[c] Millero *et al.* (1976).
[d] Kamiyama & Gekko (1997).
[e] Hayashi *et al.* (1996); lyozyme concentration 0.44 % (w/v).
[f] Chalikian *et al.* (1996b).
[g] Lee & Timasheff (1974).
[h] Gavish *et al.* (1983).

partial specific volume is almost independent of GdnCl concentration within the experimental error, the partial specific isentropic compressibility slightly decreases (about -0.03 cm^{3}g^{-1}GPa^{-1}) around the transition region.

Pressure-assisted unfolding of lysozyme was investigated in the following way. Assuming a two-state transition by compression from native to unfolded state, the equilibrium constant, K_u, and hence the Gibbs free energy change, ΔG_u, were calculated using the equation:

$$\Delta G_u = -RT \ln K_u = -RT \ln\{(X - X_n)/(X_u - X)\} \quad (6)$$

where R is the gas constant, T the absolute temperature, and X_n, X_u and X the respective differential absorption, $d\varepsilon/d\lambda$, at 290 nm for the native state (pre-transition region), for the unfolded state (post-transition region) and in the transition region, respectively. The $d\varepsilon/d\lambda$ value was calculated from the intensity, $dA/d\lambda$, at 290 nm of first-order differential absorption spectra of lysozyme. The effect of pressure on the $d\varepsilon/d\lambda$ values in various GdnCl solutions are shown in Figure 3(a), which represents the change in fraction of native or unfolded states in the transition region. Then, we can calculate the ΔG_u values by using equation (6), and the results are given in Figure 3(b). The volume change of transition, ΔV_u, is given by the following thermodynamic relation:

$$(\partial \Delta G_u/\partial p)_T = \Delta V_u \quad (7)$$

where p is the pressure applied. By assuming the linear dependence of ΔG_u on pressure in the transition region, the ΔV_u values were estimated and summarized in Table 3. The volume change of lysozyme for the pressure-assisted unfolding in GdnCl solution is negative but very small (about $-50 \sim -60$ cm^3 mol^{-1}).

Table 2. Linear regression analysis for the protein concentration dependence of the apparent specific volume and isentropic compressibility of hen lysozyme

GdnCl (mol dm^{-3})	v^0 (cm^3 g^{-1})	S_v	k^0 (cm^3 g^{-1} GPa^{-1})	S_k
0	0.734(0.001)	0.4(0.2)	0.071(0.009)	−3.3(3.6)
0.44	0.736(0.001)	0.9(0.2)	0.094(0.003)	−5.1(1.2)
1.00	0.736(0.001)	0.6(0.2)	0.083(0.005)	−0.1(2.1)
1.75	0.735(0.004)	0.6(1.5)	0.100(0.006)	−2.8(2.3)
2.41	0.738(0.001)	0.2(0.3)	0.108(0.008)	−0.5(2.8)
3.14	0.731(0.001)	1.9(0.2)	0.107(0.005)	−5.3(2.1)
3.67	0.734(0.001)	1.6(0.3)	0.101(0.004)	−0.6(1.6)
4.10	0.737(0.001)	0.5(0.3)	0.095(0.004)	−2.4(1.7)
4.62	0.733(0.001)	1.4(0.5)	0.098(0.002)	−1.8(0.9)
5.35	0.738(0.001)	0.6(0.2)	0.101(0.002)	2.7(0.7)
5.89	0.727(0.001)	2.0(0.3)	0.109(0.002)	−0.2(0.9)
7.16	0.742(0.001)	0.1(0.4)	0.115(0.002)	0.1(0.7)
7.63	0.734(0.001)	1.4(0.6)	0.117(0.003)	0.1(1.4)

Numbers in parentheses denote standard deviations.

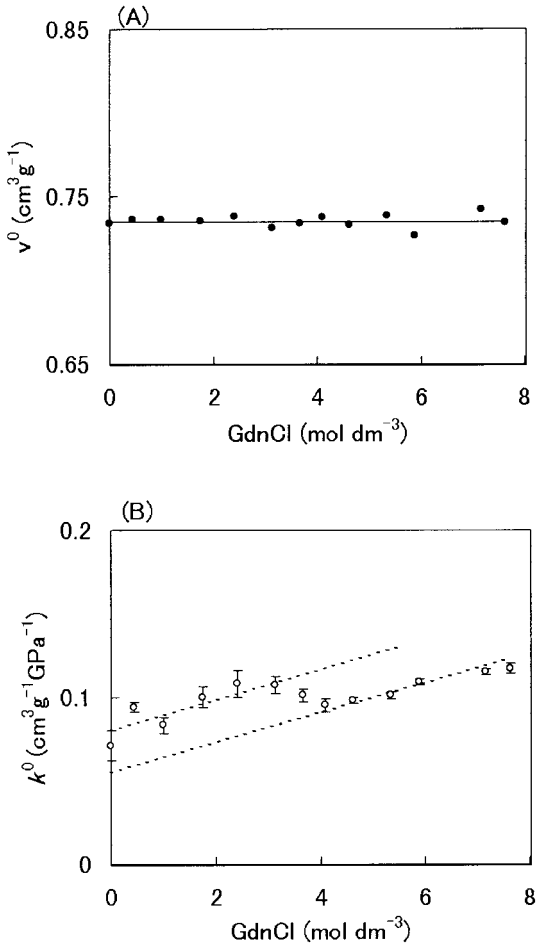

Figure 2. GdnCl concentration dependence of the (a) partial specific volumes and the (b) partial specific isentropic compressibilities of lysozyme at infinite dilution.

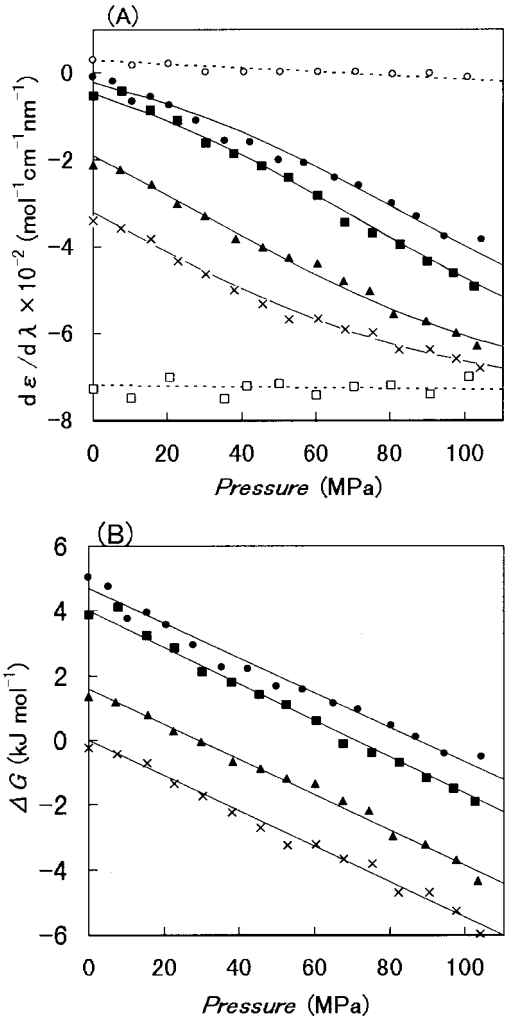

Figure 3. (a) Pressure dependence of the differential absorption at 290 nm for lysozyme in aqueous GdnCl solutions. Open circle, in 1.71 mol dm^{-3} GdnCl; filled circle, in 3.51 mol dm^{-3}; filled square, in 3.63 mol dm^{-3}; filled triangle, in 3.88 mol dm^{-3}; cross, in 4.01 mol dm^{-3}; open square, in 6.26 mol dm^{-3}. The continuous lines were calculated from the linear free energy change in (b). (b) Pressure dependence of the free energy change for lysozyme in aqueous GdnCl solutions. Filled circle, in 3.51 mol dm^{-3} GdnCl; filled square, in 3.63 mol dm^{-3}; filled triangle, in 3.88 mol dm^{-3}; cross, in 4.01 mol dm^{-3}.

Discussion

Limiting partial specific volume and isentropic compressibility of lysozyme in native state

Here, lysozyme in aqueous solution without denaturant exhibits a partial specific volume of 0.734 cm^{3}g^{-1} and a partial specific isentropic compressibility of 0.071 cm^{3}g^{-1} GPa^{-1}. As can be seen in Table 1, both of these values are slightly higher than the previous literature values except for the values reported by Millero *et al.* (1976) and Hayashi *et al.* (1996). The variation in limiting v^0 and k^0 values in Table 1 is probably due to the technical difficulty and the different experimental conditions, such as pH and salt concentration, that affect the overall hydration of a protein.

The limiting partial specific volume, v^0, of a protein can be expressed as a sum of two terms (Kauzmann, 1959; Chalikian *et al.*, 1995):

$$v^0 = v_{\text{int}} + v_{\text{h}} \tag{8}$$

where v_{int} is the specific intrinsic volume, which can be considered to the sum of two factors: (1) the geometric volume of the constituent atoms of the polypeptide chain; and (2) the void volume due to imperfect packing in the protein interior. v_{h} is the change in volume of solvent due to hydration. The intrinsic term, v_{int}, is positive, while the hydration term, v_{h}, contributes negatively to the partial specific volume of a globular protein. Based on X-ray coordinate data for 12 globular proteins, Richards (1977) has estimated the average value of the partial specific intrinsic volume of globular

Table 3. ΔV_u values calculated from the pressure dependence of the ln K_u for lysozyme in GdnCl solutions at 25 °C

GdnCl (mol dm^{-3})	ΔV_u (cm^3 mol^{-1})
3.51	−54 (8)
3.63	−56 (8)
3.88	−55 (8)
4.01	−55 (8)

Numbers in parentheses denote standard deviations.

protein to be 0.764 cm^{3}g^{-1}. By using this value and equation (8), the hydration contribution, v_h, to the partial specific volume of lysozyme is estimated to be 4 % of the v^0 value. It is generally known that the v_h contribution to the v^0 values of globular proteins, which, on average, exhibit partial specific volumes of 0.72(±0.03) cm^{3}g^{-1} at 25 °C, is about 3 to 7 % (Kunts & Kauzmann, 1974; Gekko & Hasegawa, 1986; Chalikian *et al*., 1995).

The partial specific isentropic compressibility, k_0, for globular proteins, is determined by a positive intrinsic contribution, k_{int}, and by a negative hydration contribution, k_h, (Gekko & Noguchi, 1979; Gekko & Hasegawa, 1986; Kharakoz & Sarvazyan, 1993; Chalikian & Breslauer, 1996a):

$$k^0 = k_{int} + k_h \quad (9)$$

The intrinsic contribution, k_{int}, reflects the compressible atomic contacts due to imperfect packing in the solvent-inaccessible interior of proteins. The hydration contribution, k_h, depends on the difference in compressibility between water in the hydration shell and bulk water. The hydration of protein surfaces is known to significantly decrease the compressibility of solution at room temperature because of the compact packing of water molecules in the hydration shells (Gekko & Noguchi, 1979; Kharakoz & Sarvazyan, 1993). These two contributions, k_{int} and k_h, should contain a relaxation term in the protein interior and in the hydration shell. However, this term can be usually neglected at neutral pH (Sarvazyan & Hemmes, 1979; Chalikian *et al*., 1994). The compressibility behavior is believed to be more sensitive to hydration phenomena rather than volume (Høiland, 1986; Chalikian *et al*., 1994). In fact, Figure 2(a) and (b) reveal that the increase in the GdnCl concentration leads to an increase in k^0 for the native state (pre-transition region) and for the unfolded state (post-transition region), while v^0 is almost constant.

Volume change of lysozyme for GdnCl-induced unfolding

Volumetric behavior for the unfolding of hen egg white lysozyme has been studied directly by means of densimeter or dilatometer, and indirectly by the pressure dependence of free energy change for protein stability. For instance, Lee & Timasheff (1974) reported a slightly positive volume change for GdnCl-induced unfolding of this protein by means of a densimeter, but this volume change is very small and within experimental error (30(±40) cm^3 mol^{-1}). Skerjanc & Lapanje (1972) estimated the volume change of lysozyme upon unfolding by GdnCl to be −54 cm^3 mol^{-1} at 25 °C and at pH 5.2 from their dilatometric measurements. More recently, Kamiyama & Gekko (1997) studied the unfolding of lysozyme caused by the addition of GdnCl by means of a densimeter, and reported a very large volume change of −1100 cm^3 mol^{-1} (i.e. 0.08 cm^3 g^{-1}). However, such a large negative value seems not to be reasonable to us. Careful analysis of our density measurements shows that the native and unfolded proteins give almost the same partial specific volume within the experimental error of 0.01 cm^3 g^{-1} (see Figure 2(a)). This has been also established in our previous report (Hayashi *et al*., 1996). Furthermore, the ΔV_u values for the pressure-assisted unfolding of lysozyme are only −50 to −60 cm^3 mol^{-1}, that is, −0.003 to −0.004 cm^3 g^{-1}, which are within the experimental uncertainty in the partial specific volume determined by using a densimeter.

A similar negative volume change accompanying pressure-induced unfolding of lysozyme has been reported in literature; for instance, Li *et al*. (1976) obtained $\Delta V = -19.7$ cm^3 mol^{-1} at pH 7.6 and 23 °C in their studies on pressure-induced unfolding by using the fluorescence technique. Samarasinghe *et al*. (1992) reported $\Delta V = -10.5$ cm^3 mol^{-1} at pH 3.9 and 68.5 °C by high-resolution proton magnetic resonance spectroscopy. These values are less negative than our value of −50 to −60 cm^3 mol^{-1}. This discrepancy does not appear too unsatisfactory, taking into account that our study was carried out in aqueous solutions with strong denaturant. Thus, we conclude that the change in the partial specific volume associated with GdnCl-induced unfolding is very small and is hardly detectable with sufficient precision by the densimetric measurement. This result coincides with the conclusion by Kharakoz (1997), who explained that the native and the unfolded proteins give almost the same partial volumes on the basis of an empirical additivity method for calculation of the volume change for protein unfolding.

The structural origin of the decrease in volume for protein unfolding has remained a puzzle for the last several decades (Kauzmann, 1987; Chalikian & Breslauer, 1996b; Frye & Royer, 1998). The analysis of volumetric data for a number of low molecular mass compounds has revealed that the hydration of charged and polar groups causes a decrease in volume, and the volume change on transfer of non-polar groups from hydrophobic environment to solvent water is also negative (Kauzmann, 1959; Gross & Jaenicke, 1994). In addition, the loss of void volume due to imperfect packing in the protein interior may also cause a decrease in volume. Moreover, the addition of

GdnCl may further complicate the situation due to the preferential binding of GdnCl to protein (Lee & Timasheff, 1974) and due to the structural change of water in concentrated GdnCl solution. Zipp & Kauzmann (1973) have pointed out that the experimentally observed volume change for metmyoglobin unfolding is much less negative than that expected from the volume change of a small nonpolar compound exposed to solvent water. They have concluded that the volume change for protein unfolding does not correspond to the transfer of non-polar model compounds from a nonpolar environment into aqueous solution (Kauzmann, 1987). To rationalize this volume puzzle on protein unfolding, Chalikian & Breslauer (1996b) have proposed an approach on volume changes for protein unfolding that involves changes in the solvent-accessible surface area. They suggested that the decrease in volume due to the increase in protein hydration and the loss of internal void volume in native structure is compensated by a positive contribution from the thermal volume which results from thermally induced mutual molecular vibrations of the solute and solvent.

Based on scaled particle theory calculation, Klapper (1971) proposed that small, negative volume changes for protein unfolding arise from the fact that protein interiors are very tightly packed, unlike non-polar liquids, and thus exposure of hydrophobic residues should result in a positive volume change. Frye *et al*. (1996); Frye & Rayer (1998) have suggested that the negative volume change due to exposure of charged surface is nearly compensated by a positive contribution due to the exposure of the non-polar surface, and the loss of internal void volume in the structured chain causes a major contribution to the volume change for protein unfolding. In any event, it seems that the volume change for protein unfolding can arise from a variety of origins that include contributions of opposite sign, and the experimental volume change for the unfolding of hen lysozyme does not take a value below -100 cm^3 mol^{-1} (i.e. -0.007 cm^3 g^{-1}).

Compressibility change of lysozyme for GdnCl-induced unfolding

It is known that the partial specific isentropic compressibility of many globular proteins is mostly positive, resulting from the compressible atomic contacts due to an imperfect packing in the interior of native structure. The intrinsic contribution, k_{int}, predominates over the hydration term, k_h, in equation (9) (Gekko & Noguchi, 1979; Gekko & Hasegawa, 1986). The intrinsic contribution increases as the internal packing of a globular protein loosens, but is reduced almost to zero for the completely unfolded state (Chalikian & Breslauer, 1996a). In fact, Nölting & Sligar (1993) found that the molten-globule state (a softer structure) of cytochrome *c* at pH 2.2 and 200 mM NaCl has slightly larger compressibility than the native structure. Gekko and co-workers (Tamura & Gekko, 1995; Kamiyama & Gekko, 1997) found that the GdnCl-induced unfoldings of ribonuclease A and hen egg white lysozyme are accompanied by large decreases of -0.15 to -0.16 cm^3 g^{-1} GPa^{-1} in compressibility. They have interpreted these large decreases in compressibility in terms of the loss of imperfect packing (cavity) in the native structure and the exposure of a large number of amino acid residues to solvent water. According to the empirical additivity scheme of isentropic compressibility of extended oligopeptides and polypeptides, the isentropic compressibility of a fully unfolded protein has been reported to be a negative value of about -0.12 cm^3 g^{-1} GPa^{-1} (Kharakoz, 1997). It is noted that the compressibility reduction associated with GdnCl-induced unfolding is in agreement with the theoretical value for the completely unfolded state by Kharakoz's (1997) additivity scheme of isentropic compressibility. As can be seen in Figure 2(b), however, our results show that the transition caused by the addition of GdnCl is accompanied by a small decrease of -0.03 cm^3 g^{-1} GPa^{-1} in compressibility, in spite of the breaking of the tertiary and secondary structures.

As shown in Figure 3(b), the ΔG_u values for the pressure-assisted unfolding in GdnCl solutions decrease almost linearly with pressure in the range of 0.1 to 110 MPa. Therefore, we calculated the ΔV_u values from the slope by assuming that ΔV_u is independent of pressure. This assumption appears to be reasonable if we consider that a reliable estimation of isothermal compressibility is in general very difficult, and that the isentropic compressibility, which can be estimated more accurately from sound velocity measurement, shows only a slightly negative change as seen in Figure 2(b). Taking into account that the compressibility behavior is sensitive to the interaction between the surface atomic groups of protein and the surrounding water molecules (Høiland, 1986; Chalikian *et al*., 1994), a large decrease in the partial specific compressibility for GdnCl-induced unfolding would be expected due to the solvent exposure of buried residues. The present results, however, show that this is not really the case. This compels us to suspect the notion that a protein structure is almost completely unfolded by GdnCl and a large number of amino acid residues in the protein interior are exposed to solvent water.

As early as 1970 Brandts *et al*. (1970) presaged that the volume and compressibility changes during unfolding are not dominated by the hydrophobic effects which are described by the transfer reactions of non-polar model compounds from a non-polar environment into aqueous solution. Moreover, it should be noted that an important question is to what extent proteins are unfolded at high concentrations of strong denaturants like GdnCl or urea. Some investigators have argued that the unfolded state is not necessarily modeled by a random-coil polypeptide chain (Dill & Shortle, 1991; Shortle, 1996; Pace *et al*., 1990;

Sosnick & Trewhella, 1992; Fink *et al*., 1994; Buck *et al*., 1994; Chen *et al*., 1996). In fact NMR data have provided a direct evidence for residual structure which forms a hydrophobic cluster even in an unfolded protein at a high concentration of urea (Neri *et al*., 1992). Since the compressibility behavior is determined by a positive contribution, k_{int}, and a negative contribution, k_h, the small decrease in compressibility for GdnCl-induced unfolding suggests that the polypeptide chain still has a large degree of structure in GdnCl solution. In other words, a solvent-inaccessible core, probably mostly hydrophobic, is still preserved in the GdnCl-induced unfolded state. This is consistent with the current view that the unfolded state is not necessarily modeled by a random-coil polypeptide chain.

Chalikian *et al*. (1995, 1996b) reported that the acid and base-induced transitions of cytochrome c are accompanied by small decreases of -0.03 to -0.07 $cm^3\ g^{-1}\ GPa^{-1}$ in compressibility. They have interpreted this small decrease in compressibility as being a result of the transition from the native state to the partially unfolded state of a globular protein. Based on their analysis, when the native state of globular protein is converted into the partially unfolded state, a substantial increase in solvent-accessible surface area corresponds to about 70 % of the solvent-accessible surface area expected for a fully extended chain. As demonstrated in the present study, the decrease in compressibility associated with GdnCl-induced unfolding is comparable to that arising from the transition from the native state to the partially unfolded state which was presented by Chalikian *et al*. Thus, such a small decrease of -0.03 to -0.07 $cm^3\ g^{-1}\ GPa^{-1}$ in partial specific compressibility may be typical for the transition from the native state to the unfolded state of globular proteins. With this in mind, we shall pay attention to the result reported by Lee (1991), who estimated that the solvent-accessible surface area in the unfolded state of a globular protein is no more than about two-thirds that of the completely extended polypeptide chain. More comprehensive compressibility data concerning the unfolded state will be essential for understanding the participation of water on protein unfolding.

Materials and Methods

Materials

Hen egg white lysozyme (crystallized six times) was purchased from Seikagaku Corporation, Ltd, Tokyo, Japan, and was used without further purification. The concentration of lysozyme in the sample solution was determined by weighing the component or by using an extinction coefficient (26.5) at 280 nm for a 1 % solution in a 1 cm cell. Biochemical-grade GdnCl was purchased from Wako Pure Chemical Industries., Ltd, Osaka, Japan. The concentration of GdnCl was determined by the difference between the refractive index of a GdnCl solution and that of water (Nozaki, 1972). Distilled and deionized water was used for preparing solutions.

Density and sound velocity measurements

The solution densities, d, were measured using an oscillating-tube densimeter (Anton Paar DMA60/601), which was described elsewhere in detail (Sakurai & Nakagawa, 1982; Sakurai, 1987). The temperature of the densimeter cell was maintained at 25 °C by circulating water from a constant-temperature bath controled within 0.002 deg. C. The densimeter was calibrated with water and dry air every day. The density could be determined with a precision of $\pm 2 \times 10^{-6}$ g cm^{-3}.

The sound velocities, u, in solution were determined by using a homemade sing-around velocimeter operating at 5 MHz. The cell of the velocimeter was placed deep in the water-bath used for the density measurements. The precision of sound velocity measurements was estimated to be better than 1 cm s^{-1} for the dilute solution range studied. Details of the apparatus, its calibration, and experimental procedures used have been described (Sakurai *et al*., 1995).

The evaluation of the reliable value of apparent specific volume or isentropic compressibility in dilute solutions requires very accurate determinations of the difference in density and/or sound velocity between solution and solvent. It is rather difficult to fill this experimental requisite in chemical unfolding of protein studies, since we are forced to use a concentrated denaturant solution as a solvent, where even a very little variation in the solvent composition is not permissible during a series of measurements. In this study, therefore, we carefully prepared the sample solution as follows: A large quantity of solvents, GdnCl solutions ($0 \sim 7.6$ M) without any buffer, were prepared in order to minimize the uncertainty due to the composition change mentioned above. Then a stock solution of about 0.6 % (w/w) was made up by dissolving lysozyme in a given GdnCl solution before a series of the density or sound velocity measurements. The protein solutions were prepared by successive addition of the stock solution into the measuring cell containing a known quantity of the solvent. The six or seven additions were carried out by mass and the concentration of the solution finally reached to about 0.3 % (w/w).

Circular dichroism measurements

The GdnCl-induced unfolding of lysozyme was confirmed by CD measurements with a Jasco J-725A spectropolarimeter. Ellipticities at 289 nm and at 222 nm were measured in a quartz cell with 10 mm and 1 mm path lengths, respectively. The concentration of lysozyme was 0.04 % (w/w). The temperature surrounding the cell was maintained by circulating an ethylene glycol(water mixture from a constant-temperature bath controlled within 0.1 deg. C.

Pressure-assisted unfolding

The pressure-assisted unfolding of lysozyme in aqueous GdnCl solutions was detected by UV measurements with a Shimadzu UV-3000 spectrophotometer. A high-pressure vessel (Hikari High-Pressure Instruments, Ltd., Hiroshima, Japan) made of high-tensile stainless steel with two sapphire windows was specially designed to fit into the spectrophotometer. The sample cell for the measurement was made of quartz with 10 mm path length and with a capillary tube of 2 mm diameter. The quartz cell, filled with an aqueous protein solution, was

fixed into the high-pressure vessel by a cell holder made of brass. The capillary of the cell was sealed with mercury in order to separate the sample solution from the pressure mediator (2,2,4-trimethylpentane). The temperature of the pressure vessel was controlled within 0.1 deg. C by circulating an ethylene glycol + water mixture from a constant temperature bath. The temperature was monitored *via* a thermometer installed inside the pressure vessel.

Pressure was regulated with a hand-pump over a range of 0.1 to 110 MPa, and was transmitted to the sample solution through a stainless steel tube containing mediator. The pressure of the sample was increased by 7-10 MPa steps and was read with a Heise pressure gauge. After ten minutes, the intensity, $dA/d\lambda$, at 290 nm of the first-order differential absorption spectra from 292 to 286 nm was measured as an equilibrium value. This $dA/d\lambda$ value was transformed into the $d\varepsilon/d\lambda$ value. The measurements were repeated seven times and the average $d\varepsilon/d\lambda$ value was recorded. The concentration of protein solution was 0.04% (w/w), and all measurements were carried out at 25°C and under neutral pH conditions.

Acknowledgments

We thank Dr Nobuo Takenaka for constructing the sing-around velocimeter. We also would like to express our appreciation to Professor Mitsuo Nakata for constructing the pressure generation system.

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Edited by P. E. Wright

(Received 9 February 1999; received in revised form 25 May 1999; accepted 28 June 1999)